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Exoglucanase Activities of the Recombinant *Clostridium thermocellum* CelS, a Major Cellulosome Component

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The recombinant CelS (rCelS), the most abundant catalytic subunit of the *Clostridium thermocellum* cellulosome, displayed typical exoglucanase characteristics, including (i) a preference for amorphous or crystalline cellulose over carboxymethyl cellulose, (ii) an inability to reduce the viscosity of a carboxymethyl cellulose solution, and (iii) the production of few bound reducing ends on the solid substrate. The hydrolysis products from crystalline cellulose were cellobiose and cellotriose at a ratio of 5:1. The rCelS activity on amorphous cellulose was optimal at 70°C and at pH 5 to 6. Its thermostability was increased by Ca^{2+}. Sulfhydryl reagents had only a mild adverse effect on the rCelS activity. Cellotetraose was the smallest oligosaccharide substrate for rCelS, and the hydrolysis rate increased with the substrate chain length. Many of these properties were consistent with those of the cellulosome, indicating a key role for CelS.

Clostridium thermocellum, an anaerobic and thermophilic bacterium, produces an active extracellular cellulase system (14) called the cellulosome (19). The cellulosome is an extremely complicated protein complex consisting of more than 14 subunits with a total molecular weight in the millions (7, 19). Studies of the structure-function relationship of the cellulosome had long been plagued by the complexity of the cellulosome's structure. Recently, exciting progress has been made. It has become clear that the cellulosome uses a novel mechanism in degrading crystalline cellulose (2, 31). The newly proposed one-anchor–multiple-enzyme model involves catalytic subunits lining up in an array on a scaffolding and anchorage subunit, CipA (formerly CelL or S_L [11]). Among those catalytic subunits, CelS (formerly S_S) plays an important role. It is the most abundant catalytic subunit in the cellulosome (31) and has been reported to degrade crystalline cellulose synergistically with CipA (CelL) in an enzyme (CelS)-anchor (CelL) manner (32, 33). The one-anchor–multiple-enzyme organization has also been found in the cellulase systems of other anaerobic bacteria such as *Clostridium cellulovorans* (for a review, see reference 8), *Clostridium josui* (10), and *Clostridium cellulolyticum* (3).

The cellulosome represents a novel class of mechanism for cellulases or even for enzymes in general. To further understand the cellulosome mechanism, it is crucial to characterize the individual cellulosome components, particularly CelS. We have recently cloned and sequenced the *celS* gene (28, 30). Although a very large number of *cel* genes have been cloned and sequenced, *celS* belongs to a new cellulase family. The *celS* gene has been expressed in *Escherichia coli*, and initial characterization of the gene product indicates that CelS is an exoglucanase (29). This report describes the confirmation of the exoglucanase characteristics of recombinant CelS (rCelS) expressed in *E. coli* and characterizations of its enzymatic properties.

Purification of rCelS. The expression of the *celS* gene in *E. coli* and purification of the gene product were performed essentially as previously reported (26, 29). In brief, the *celS* gene was expressed as inclusion bodies by using the pRSET expression vector (29). The purified inclusion bodies were solubilized in 5 M urea. The dialyzed proteins were treated at 60°C for 10 min in the presence of 10 mM $CaCl_2$ to remove the heat-labile *E. coli* proteins. A final purification step was carried out on a Resource Q ion-exchange column (Pharmacia Biotech, Piscataway, N.J.) equilibrated with 20 mM Tris-HCl (pH 8.0). The rCelS was eluted at a 150 mM salt concentration, yielding a preparation containing predominately rCelS (M_r, 86,000; [Fig. 1, lane 3]). As previously reported (29), a fusion sequence from pRSET contributed to the higher apparent molecular weight (86,000 for rCelS versus 82,000 for CelS). The rCelS was dialyzed against 20 mM Tris-HCl (pH 7.0) and used for all the experiments described below.

Substrate specificity of rCelS. The cellulase activities against carboxymethyl cellulose (CMC) (type 7L2; Hercules, Wilmington, Del.), Avicel (type PH-105, a microcrystalline cellulose; FMC, Newark, Del.), xylan (Fluka AG, Buchs, Switzerland), cotton, or amorphous cellulose (phosphoric acid-swollen cellulose [29]) were measured in 50 mM succinate buffer (pH 5.7) containing 10 mM $CaCl_2$ and 0.5% (wt/vol) (or 0.1% [wt/vol] for amorphous cellulose) substrate. The reducing sugars produced were measured (24) with glucose as a standard. The activities on *p*-nitrophenyl β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl β-D-cellobioside (*p*NPC), *p*-nitrophenyl β-D-xylopyranoside (*p*NPX), or 4-methylumbelliferyl cellobioside (MUC) were measured in 50 mM succinate buffer (pH 5.7) containing 10 mM substrate. The released *p*-nitrophenol was measured at 400 nm with a spectrophotometer after adding Na_2CO_3 to a final concentration of 60 mM. The activity on MUC was qualitatively determined by examining the fluorescence under a UV light.

The highest level of rCelS activity was observed with amorphous cellulose (Table 1). Microcrystalline cellulose (Avicel) was hydrolyzed at a much lower rate. A very low, but detectable, level of activity was observed with CMC. On the other hand, rCelS showed no activity on xylan (from either oat spelt or birch wood) or cotton under the assay conditions. Furthermore, it showed no activity toward chromogenic or fluorogenic derivatives of cellobiose (MUC and *p*NPC), glucose (*p*NPG), or xylose (*p*NPX). This explains why the *celS* gene had long

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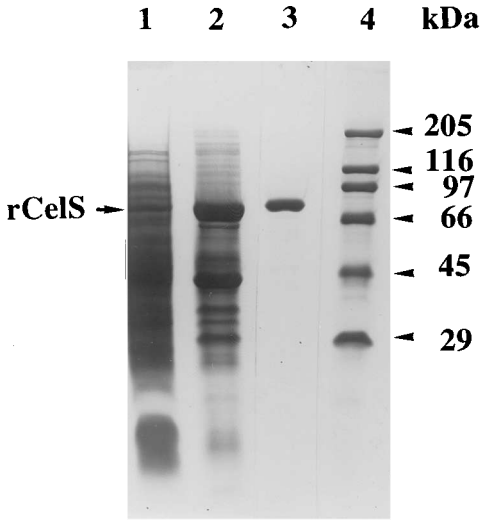

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the rCelS preparations. Lane 1, cell lysate of the *E. coli* clone harboring pKK34 (29) containing the *celS* gene (8 μg of protein); lane 2, inclusion bodies after solubilization in 5 M urea and dialysis (7 μg of protein); lane 3, purified rCelS after ion-exchange chromatography (3 μg of protein); lane 4, molecular mass markers.

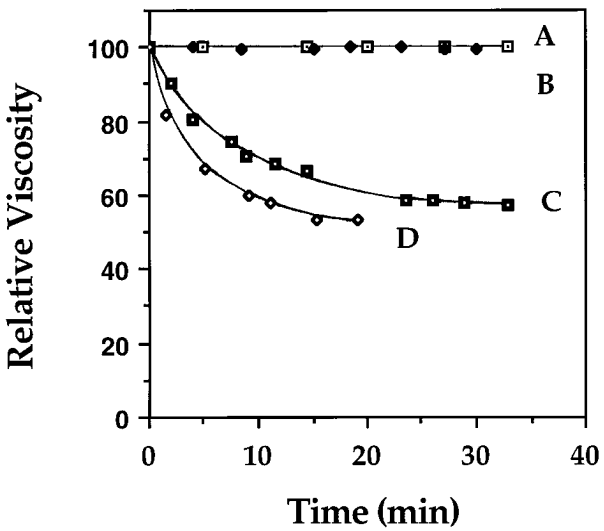

FIG. 2. Viscosimetric analysis of purified rCelS and rCelD (a known endoglucanase [16]). The reaction mixture contained 10 ml of 1% (wt/vol) CMC in 100 mM succinate buffer (pH 5.7) and 14 μg of purified rCelS (curve A, boxes with black dots), 35 μg of rCelS (curve B, solid diamonds), 2.7 μg of rCelD (curve C), or 5.4 μg of CelD (curve D). The assays were carried out at 60°C. The 100% relative viscosity represents the difference between the viscosity of the CMC solution and that of pure water. The specific activity of CelD on CMC was 29 μmol of glucose equivalents per min per mg.

escaped cloning efforts primarily with CMC or chromogenic substrates for activity screening.

Hydrolysis products. The products of enzymatic hydrolysis by rCelS were analyzed by using a high-performance liquid chromatography (HPLC) column (Aminex HPX 42A; Bio-Rad Laboratories, Hercules, Calif.) at 75°C and a refractive index detector (Perkin-Elmer, Norwalk, Conn.). Cellobiose was the major hydrolysis product from Avicel, although cellotriose was also detected with a cellobiose/cellotriose ratio of approximately 5:1. Similarly, cellobiose was the major hydrolysis product from amorphous cellulose. Small amounts of cellotriose and cellotetraose were also released. However, cellotetraose disappeared with prolonged hydrolysis (24 h), presumably because of further degradation to cellobiose. Glucose was not detected in any case.

Optimal conditions for rCelS activity. The effect of temperature on the activity of rCelS was examined with amorphous cellulose as the substrate. The optimal temperature under the assay conditions was 70°C, the same as that for the crude *C. thermocellum* cellulase (14). Ca^{2+} at 10 mM significantly enhanced the activity of rCelS only at high temperatures (>60°C), indicating that Ca^{2+} increases the thermostability of rCelS. Ca^{2+} has been reported to stimulate the activity of the *C. thermocellum* crude enzyme (14) and the purified cellulosome (18). It also increases the thermostability of the *C. thermocellum* CelD (6) and S8-tr, a truncated form of the S8 protein (23).

The effect of pH on the activity of rCelS was examined with the same substrate in 50 mM succinate (pH 4 to 6.5) or MOPS (morpholinepropanesulfonic acid) (pH 6.5 to 8) buffer containing 10 mM Ca^{2+}. The purified rCelS exhibited a pH optimum of 5.5, which is very close to the pH optimum (5.7) of the *C. thermocellum* crude enzyme (14).

Viscosimetric analysis on CMC. As mentioned above, rCelS released only a very low level of reducing sugar from CMC, while it was more active on crystalline or amorphous cellulose (Table 1). These are characteristics typical of an exoglucanase. To further characterize its mode of action, we carried out a viscosimetric assay on rCelS by using CMC as the substrate and a Cannon Ubbelohae capillary viscosimeter (Technical Glass Products, Rockaway, N.J.). A well-characterized *C. thermocellum* endoglucanase, CelD (15, 16), was used as a control. The *celD* gene was cloned into pTRXFUS (20) by PCR (25) with *C. thermocellum* genomic DNA as the template. The two PCR primers were 5′-GCGGGTACCAAGCTTGACCGGAGTATTTC-3′ (forward primer) and 3′-AGCTCTTTAATGGTTATATTGACGTCCGG-5′ (reverse primer). The *celD* gene (about 1.9 kb) obtained by PCR was fused in frame to the thioredoxin gene of pTRXFUS by using the *Kpn*I and *Pst*I cloning sites. The fused *celD* gene was expressed as described by LaVallie et al. (20). Active rCelD was found in the cytoplasms of the host cells in a soluble form. A gel electrophoresis analysis (17) indicated that the heat-treated (70°C for 1 h) cell lysate contained predominantly rCelD.

As expected, rCelD (2.7 or 5.4 μg, assayed as described in reference 4) rapidly reduced the viscosity of a CMC solution

TABLE 1. Activities of rCelS on various substrates[a]

Substrate	Amt of reducing sugar produced (μg of glucose/ml)	Sp act of rCelS (nmol of glucose/min/mg of protein)
Amorphous cellulose	57.0	150
Avicel	7.1	19
CMC	3.0	8
Xylan (oat spelt)	ND[b]	
Xylan (birch wood)	ND	
Cotton	ND	
*p*NPG	ND	
*p*NPC	ND	
*p*NPX	ND	
MUC	ND	

[a] The data are averages of two identical runs. The assay mixture contained 7 μg of purified rCelS. The assays were carried out at 60°C for 5 h (with amorphous cellulose, Avicel, CMC, xylan, and cotton) or 16 h (with *p*NPG, *p*NPC, *p*NPX, and MUC).

[b] ND, not detected.

TABLE 2. Distribution of reducing ends between Avicel (bound) and supernatant (free) after hydrolysis[a]

Enzyme	Amt of protein (μg)	% Digestion[b]	Amt of reducing sugar produced (μg/ml)[c]		Reducing sugar (%)		Soluble/insoluble (free/bound) reducing sugar ratio
			Avicel	Supernatant	Avicel	Supernatant	
rCelS	14.0	4.6	2.2	20.9	9.5	90.5	9.5
rCelD	13.5	15.2	22.1	53.5	29	71	2.4

[a] Hydrolysis times were 17 h for rCelD and 26 h for rCelS. The data are the averages of triplicate runs.
[b] Percentage digestion was calculated on the basis of the total amount of (free and bound) reducing sugar released as glucose equivalents and the substrate concentration before hydrolysis.
[c] Amounts of reducing sugar are presented as glucose equivalents.

(Fig. 2, curves C and D). In contrast, rCelS (14 or 35 μg) failed to reduce the viscosity even at much higher protein loadings (Fig. 2, curves A and B), as would be expected from an exoglucanase.

Distribution of reducing ends generated by rCelS. The major activity of an exoglucanase is to release soluble reducing sugars from crystalline cellulose into the solution. On the other hand, an endoglucanase cuts the cellulose chain randomly, leaving some reducing ends attached to the insoluble substrate. The ratio of soluble to insoluble reducing sugar has therefore been used as a criterion for distinguishing an exoglucanase from an endoglucanase (12). Typically, an exoglucanase generates 10% or less insoluble reducing ends de novo, whereas an endoglucanase generates 30 to 50% (12).

We examined de novo formation of soluble and insoluble reducing sugar from Avicel by rCelS by the method of Irwin et al. (12) except that the enzymatic reactions were carried out in 50 mM succinate buffer (pH 5.7) at 60°C and the reducing ends were determined by the ferricyanide method (24).

Under the assay conditions, about 90% of the reducing ends generated by rCelS were soluble and only about 10% remained insoluble. In contrast, rCelD produced 29% insoluble reducing ends (Table 2). These results indicate that CelS is an exoglucanase and confirm that CelD is an endoglucanase.

Hydrolysis of cellodextrins. As described above, while the major cellulose hydrolysis product by rCelS was cellobiose, a small amount of cellotriose was also observed. This suggested that CelS was not able to hydrolyze cellotriose. On the other hand, cellotetraose was only transiently produced from amorphous cellulose, suggesting that rCelS could further hydrolyze cellotetraose. To determine the activity of rCelS on cellooligosaccharides, we incubated the purified rCelS with individual cellooligosaccharides and analyzed the hydrolysis products by HPLC.

As shown in Table 3, cellobiose and cellotriose were not hydrolyzed by rCelS. Cellotetraose was degraded to cellobiose.

TABLE 3. HPLC analysis of the hydrolysis products from cellodextrins by rCelS[a]

Substrate	Presence of hydrolysis product				Hydrolysis rate (nmol of substrate/min/ml)
	Cellotetraose	Cellotriose	Cellobiose	Glucose	
Cellobiose				−	0
Cellotriose			−	−	0
Cellotetraose		−	+	−	0.32
Cellopentaose	−	+	+	−	0.78

[a] The reaction mixture, in a total volume of 200 μl, contained 3 μg of purified rCelS and 0.1% (wt/vol) cellobiose, cellotriose, cellotetraose, or cellopentaose in 10 mM succinate buffer (pH 5.7). The reaction was carried out at 60°C for 17.5 h.

Cellopentaose was hydrolyzed to cellobiose and cellotriose in an equimolar ratio. The degradation rate of cellopentaose was more than twice that of cellotetraose, indicating that CelS prefers longer-chain substrates. Glucose was not produced from any of the cellooligosaccharides tested.

Effect of sulfhydryl reagents. The Avicelase activity of the *C. thermocellum* crude enzyme is sensitive to oxygen inactivation and sulfhydryl reagents (13, 14). We examined the effects of sulfhydryl reagents on the activity of rCelS toward amorphous cellulose and found that its activity was only mildly reduced by the two sulfhydryl reagents tested in a 19.5-h reaction. The rCelS still retained 85% activity in the presence of 5.0 mM *o*-iodosobenzoate and 81% activity in the presence of 0.08 mM *p*-chloromercuribenzoate. At these concentrations, the two reagents would completely inactivate the crude enzyme (13).

The *celS* gene belongs to a new cellulase gene family. Genes homologous to *celS* have been found in *C. cellulolyticum* (1), *C. josui* (10), and *Caldocellum saccharolyticum* (21). In addition, the N-terminal sequences of the Avicelase II of *Clostridium stercorarium* (5) and the S8 protein of the YS strain of *C. thermocellum* (22) are identical or nearly identical to that of CelS. We classify CelS as an exoglucanase on the basis of the following three criteria: (i) the substrate specificities of the enzyme, (ii) the distribution of the soluble and insoluble reducing ends after enzymatic hydrolysis, and (iii) the ability of the enzyme to reduce the viscosity of a CMC solution. In addition, the observation that cellobiose was the major hydrolysis product is consistent with this classification.

CelS shares many characteristics with S8-tr, a truncated form of S8 (23). This indicates that the truncation of S8, presumably at the C-terminal end, where a conserved, duplicated sequence lies (9, 23, 27), does not significantly alter S8's enzymatic properties. However, CelS, unlike S8-tr, produced small amounts of cellotetraose and cellotriose in addition to cellobiose and was not active on xylan. The former difference may be due to the fact that the HPLC used in this work to detect the hydrolysis products is more sensitive to the low levels of oligosaccharides than is the thin-layer chromatography used by Morag et al. (23). The properties of CelS reported here are also consistent with those of Avicelase II of *C. stercorarium* (5). In particular, both proteins generate cellotriose in addition to cellobiose from Avicel and transiently generate cellotetraose from amorphous cellulose. It is noteworthy that Avicelase II is a free enzyme, while CelS belongs to a large protein complex (the cellulosome). Like S8-tr, Avicelase II is also active on xylan. However, Bronnenmeier et al. (5) suspected that this may be due to a small amount of xylanase contamination.

Many of the enzymatic properties of rCelS reported here are similar to those of the crude enzyme (14). The similarities include (i) the production of cellobiose as the major hydrolysis product, (ii) activity enhanced by Ca^{2+}, (iii) a pH optimum between 5 and 6, and (iv) a temperature optimum of 70°C.

These similarities indicate that CelS plays an important role in the cellulolytic activity of the crude enzyme. In sharp contrast to the crude enzyme, rCelS was only mildly inactivated by sulfhydryl reagents. This indicates that the sulfhydryl reagents do not modify the amino acid residue(s) involved in the intrinsic enzymatic activity of rCelS. However, the sulfhydryl reagents may adversely modify the CelS amino acid residue(s) involved in the protein-protein interaction crucial for its synergism with another cellulosome component(s). Alternatively, the sulfhydryl reagents may inactivate another crucial component(s), such as CipA. Our finding that CelS is an exoglucanase is consistent with the general understanding that an exoglucanase is crucial for degrading crystalline cellulose and further confirms its significant role in the cellulosome. Its physiological importance may therefore explain its abundance in the cellulosome. The enzymatic properties of CelS reported in this work will be useful for further elucidating the mechanism of the cellulosome.

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